AD NUMBER

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AD_____

GRANT NUMBER DAMD17-96-1-6149

TITLE: Control of Breast Tumor Cell Growth by Dietary Indoles

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CONTRACTING ORGANIZATION: University of California
Berkeley, California 94720-5940

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Control of Breast Tumor Cell Growth by Dietary Indoles

5. FUNDING NUMBERS
DAMD17-96-1-6149

6. AUTHOR(S)
Leonard Bjeldanes, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Berkeley, California 94720-5940

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Indole-3-carbinol (I3C), a naturally occurring component of Brassica vegetables has been shown to reduce the incidence of mammary tumors. Our studies of the molecular mechanisms and signal transduction pathways by which I3C mediates its antiproliferative effects have established that I3C can induce a reversible G1 cell cycle arrest of cultured human breast cancer cells (MCF7 cells) in the absence of estrogen receptor signaling. This cell cycle arrest is accompanied first by a rapid inhibition of expression of the cyclin dependent kinase-6 (CDK6) cell cycle component and later by a stimulation in production of both the p21 and the p27 CDK inhibitors. Importantly, these I3C-mediated effects functionally alter the ability of G1-acting CDK2 and CDK6 to phosphorylate the Rb tumor suppressor protein, which is a necessary event for cell cycle progression. We have also shown that while I3C products possess significant cytostatic activity against the estrogen-induced growth of MCF7 cells, their activities in estrogen-responsive reporter genes are highly dependent on the gene construct, suggesting that the indoles do not function as classical estrogen antagonists in these studies. Instead, the indoles may function by activation of other genes central to the regulation of estrogen-induced cellular responses.

14. SUBJECT TERMS
antiestrogen, estrogen receptor, gene regulation, dietary indoles, Ah receptor, cell cycle arrest Breast Cancer

15. NUMBER OF PAGES
62

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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Leonard Bjeldanes 9/26/97

PI - Signature Date

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Introduction:

Indole-3-carbinol (I3C) is a naturally occurring component of dietary vegetables and a promising cancer preventive agent, most notably against breast cancer. I3C markedly reduces the incidence of spontaneous and carcinogen-induced mammary tumors in rodents and exhibits potent growth inhibitory activity in human breast cancer cells. Although I3C has reached the stage of phase I clinical trials, little is known about the mechanism of its growth inhibitory effects in cancer cells. The purpose of this work is to establish the mechanism of action and to exploit the cancer preventive properties of I3C and related compounds.

I3C is active in several key anticancer-related bioassays. Rodents exposed to high doses of I3C via oral intubation or diet exhibited increases in the activities of a variety of cytochrome P-450-dependent activities including hepatic ethoxyresorufin O-deethylase (EROD) and related activities (1,2). I3C reduced BP-induced neoplasia of the forestomach (3) and total covalent binding of BP and N-nitrosodimethylamine to hepatic DNA in mice (4,5,6). In trout, I3C reduced AFB_1-induced hepatocarcinogenesis when administered prior to and during carcinogen treatment (7). In a recent screen of 90 potential chemopreventive agents in a series of 6 short term bioassays relevant to carcinogen-induced DNA damage, tumor initiation and promotion, and oxidative stress, I3C was found to be one of only 8 compounds that tested positive in all assays. The authors of this study opined that I3C was highly promising for development as a cancer chemopreventive agent (8).

Some of the most well established biological effects of I3C appear to be related to its antiestrogenic effects. In a long term feeding experiment, in which female mice consumed synthetic diets containing I3C at 0, 500 or 2000 p.p.m., spontaneous mammary tumor incidence and multiplicity were significantly lower (ca. 50% reduction) at both doses of I3C than for untreated control animals, and tumor latency was prolonged in the high dose group (9). Oral administration of I3C to humans at doses of around 500 mg daily for one week produced an increase in estradiol 2-hydroxylation of approximately 50% in both men and women (10). I3C also increased the levels of estradiol hydroxylation activity in female rats (11).

The effects of I3C on DMBA-induced mammary tumors in rodents were reported in two studies. Wattenberg reported that I3C administered in the diet or by oral intubation prior to treatment with carcinogen reduced tumor incidence by 70-80% (3). In a recent study by Lubet, I3C administered prior to and during DMBA treatment reduced mammary tumor incidence by as much as 95% in rats (12). In a post-initiation protocol, I3C administration following treatment with NMU reduced tumor incidence by 65% (12). Consistent with these results, supplementation of a purified diet with cabbage or broccoli, both of which vegetables are good sources of I3C, also resulted in decreased mammary tumor formation in DMBA-treated rats (13).

The antiestrogenic growth suppressive effects of I3C are also established for breast tumor cells in culture. Estradiol-induced proliferation of high density human MCF-7 cells was totally blocked by 50 μM I3C in the growth medium. I3C did not affect the proliferation of the estrogen-independent breast tumor line MDA-MB-231 in this experiment (14).

Because of these well documented cancer protective effects of I3C, along with its effects on estrogen metabolism, its low toxicity, and its wide

availability, I3C is currently undergoing at least two different phase I clinical trials as a cancer chemotherapeutic and preventive agent (12).

Our working hypothesis is that I3C stimulates changes in the expression and activity of a network of early-response regulatory molecules that control a subsequent cascade of events leading to the arrest of human mammary tumor cell growth. We suggest further that one result of activation of this cascade is the blockade of estrogen-induced signal transduction pathways central to cell growth and proliferation at high cell density, whereas at low cell density I3C acts in an estrogen receptor independent manner.

Body:

1. Results and discussion

During this initial period of the grant we established the experimental basis for accomplishing the major objectives as originally stated. Key pieces of laboratory equipment and supplies were obtained and we have established reliable conditions for studies of the estrogen-responsive proliferation of specific isolates of MCF7 cells in culture. We have also established conditions for high efficiency transfection of the breast tumor cells and the sensitive assay of estrogen induced reporter gene activities. Progress was made under each of the stated objectives and a manuscript describing the estrogen-independent growth cell cycle effects of I3C has been submitted for publication.

I. Identify I3C products that are responsible for I3C's growth inhibitory effects in breast tumor cells. Results of our initial studies of the antiproliferative effects of I3C and its products indicate that the dimeric indole product (DIM), the indole reaction mixture (RXM), as well as ICZ and I3C are effective cytostatic agents in the MCF7 human breast cancer cell line.

Further studies of the growth inhibitory effects of I3C in breast tumor cells, indicated that at low concentrations only the estrogen dependent growth is inhibited, while at high doses estrogen independent growth is almost completely blocked, as well.

In addition to retarding cell proliferation, as indicated by cell counting, I3C strongly inhibited [^{3}H]thymidine incorporation in MCF7(JB) cell cultures in a dose dependent manner. Cell morphology was changed at the highest concentration to a more elongated phenotype. Time course studies of I3C addition and withdrawal demonstrated that the I3C mediated growth suppression is completely reversible demonstrating that this compound does not affect cell viability. Prolonged exposure (5 days) to I3C did not result in any detectable cell death. Flow cytometry profiles of propidium iodide stained nuclear DNA revealed that I3C induces an apparent cell cycle arrest of these breast cancer cells. I3C treatment altered the DNA content of the MCF7 cell population from an asynchronous population of growing cells in all phases of the cell cycle to one in which most (72%) of the I3C treated cells exhibited a 2n DNA content, which is indicative of a G1/G0 block in cell cycle progression. Similar studies of the cytostatic effects of I3C on the estrogen independent breast tumor cell line MDA-MB-231, indicated that only the higher doses of I3C (> 100 μM) were effective.

II. Characterize effects of I3C products on estrogen receptor- and

Ah receptor-mediated cellular responses.

a. Estradiol metabolism. In our initial efforts to examine the effects of indoles on Ah receptor mediated E2 metabolism in MCF7 cells, we observed, using GC/MS analysis, that treatment of cells with I3C resulted in a nearly 60% reduction in E2 level in the medium after 24 h compared to the controls treated only with the DMSO solvent vehicle. This result is most striking particularly in light of our observation that the activity of the CYP1A1 enzyme, which is of established importance in E2 metabolism in MCF7 cells and a diagnostic indicator of Ah receptor-mediated gene induction, is not affected by the concentration of I3C used in these studies. In work planned for the next year of the project, we will identify the metabolites of estrogen produced in response to the indoles, examine the role of the Ah receptor in these metabolic effects.

b. Estrogen induced reporter genes. Mechanistic studies of the antiestrogenic effects of I3C have begun with the analysis of effects on estrogen induced reporter gene activities. We initially established reliable conditions for high efficiency transfection of reporters and analysis of CAT activity. Estrogen receptor responsiveness was monitored by transient transfection of an ERE-vit-CAT reporter plasmid containing two consensus estrogen response elements linked to the vitellogenin promoter driving the bacterial chloramphenicol acetyl transferase gene. In cells transfected by the lipofectamine procedure, reporter gene activity was assayed by monitoring the conversion of [^{3}H]acetyl-CoA (plus unlabeled chloramphenicol) into [^{3}H]acetylchloramphenicol using a quantitative, non-chromatographic extraction procedure we have optimized. We observed that I3C and ICZ can inhibit estrogen induced CAT expression in this system by up to about 50%, and that DIM and RXM are less active. Our continuing work in this area will examine the effects and modes of the indoles in other estrogen- and AP-1-responsive reporters.

c. Ah receptor deficient MCF7 cells. Antisense AhR transfection was used to create an AhR deficient cell line. Ten monoclones were obtained from this cell line. These clones were initially characterized by EROD assay, western blot and growth rate. Compared to the control cells, which are transfected by the plain vector without antisense AhR cDNA, the EROD activity of monoclones aAhR4 and aAhR8 was over 80% inhibited. Western blot showed that very little expression of AhR in aAhR4 cell, however, aAhR8 showed the normal expression of AhR. The full characterization of response of these cells to indoles is in progress.

III. Identify genes involved in the I3C-mediated inhibition of growth of breast tumor cells.

a. Cell cycle effects. In studies of the cell cycle effects of I3C during the past year we have established that I3C can induce a reversible G1 cell cycle arrest of MCF7 cells in the absence of estrogen receptor signaling. This cell cycle arrest is accompanied first by a rapid inhibition of expression of the cyclin dependent kinase-6 (CDK6) cell cycle component and later by a stimulation in production of both the p21 and the p27 CDK inhibitors. No changes were observed in expression of the other G1-acting CDKs (CDK2 and CDK4) as well as on the G1-acting cyclins. Importantly, these I3C-mediated effects on the expression of specific cell cycle components functionally alter ability of G1-acting CDK2 and CDK6 to phosphorylate the Rb tumor

suppressor protein, which is a necessary event for cell cycle progression, is strongly suppressed by I3C treatment. In our studies with human MCF7 breast cancer cells, both time course and dose response assays with I3C revealed that the loss in CDK6 production closely coincided with the reduction in cell proliferation which implicates CDK6 as a direct target for cell cycle control in human breast cancer cells. The rapid I3C effect on CDK6 gene expression is unique because it is the first report of a growth inhibitor decreasing the mRNA for CDK6. Within fifteen hours of I3C treatment, there is a significant reduction in CDK6 transcript levels which likely accounts for the decrease in CDK6 protein. To directly test this concept, CDK6 was over-expressed in MCF7 cells by transfection of a CDK6 expression vector. Preliminary evidence shows that MCF7 cell lines constitutively expressing CDK6 fail to undergo the I3C-mediated cell cycle arrest. Thus, our observations have uncovered a previously undefined antiproliferative pathway for I3C that implicates CDK6 as a target for cell cycle control in human breast cancer cells. The selective regulation of CDK6 expression in mammary epithelial cells by I3C provides the basis to propose a biological mechanism by which I3C potentially controls the emergence and proliferation of breast cancer cells.

The I3C-mediated cell cycle arrest and repression of CDK6 production was observed in ER deficient MDA-MB-231 human breast cancer cells under conditions in which the antiestrogen tamoxifen had no effect on cell growth. In ER containing MCF7 cells, tamoxifen suppressed DNA synthesis to approximately the same extent as I3C, but had no effect on CDK6 expression. Moreover, while tamoxifen reduced the activity of an ERE-containing reporter plasmid in cells cultured with FBS containing endogenous estrogens, I3C had no effect on ER responsiveness under these same culture conditions. Consistent with I3C and tamoxifen acting through distinct antiproliferative pathways, a combination of I3C and tamoxifen inhibited MCF7 cell growth to a greater extent compared to the effects of either agent alone. Taken together with previous studies on the acid-catalyzed products of I3C, our results with I3C indicate that dietary indoles are likely to work through both ER-independent and ER-dependent pathways. For the ER-independent pathway, I3C suppresses breast cancer cell growth by a rapid inhibition of CDK6 expression and a later stimulation of CDK inhibitor production. We propose that this effect causes an inhibition of the activity of G1-acting CDKs and thereby induces a G1 block in cell cycle progression. We also propose that I3C is mediating these effects through a putative cellular receptor. In contrast, the ER-dependent pathway is mediated by the I3C acid-catalyzed product ICZ (indolo-[3,2-b]carbazole) which binds to and activates the aromatic hydrocarbon receptor (AhR). The AhR transcriptionally activates cytochrome P4501A1 (CYP1A1)-dependent monooxygenase which inactivates estrone and thereby prevents the estrogen stimulated growth of breast cancer cells. Our results suggest that I3C, in combination with tamoxifen, could prove to be an effective therapy. Patients could receive intermittent pulses of tamoxifen or lower doses of tamoxifen, both of which are proposed methods of circumventing tamoxifen resistance, while on I3C treatment. I3C has been shown to reduce the formation of both spontaneous and carcinogen-induced mammary tumors in rodents with no apparent side-effects and human subjects treated with I3C also had no side-effects.

The key goals for the next year of grant support will be to understand the

precise molecular interactions of cell cycle components that are altered by I3C and determine the mechanism by which I3C down regulates CDK6 expression. We also plan to determine the role of I3C-regulated cell cycle components in the in vivo control of MCF7 cell-derived tumor formation.

b. Identification of new indole responsive genes. Thirty-two candidate genes were obtained from the RNA differential display of the MCF-7 JB cells with treatment of indoles and estrogen. All these genes were also sequenced and had the homology search done in the Gene Bank database. Nonradioactive northern hybridization analysis was developed in this lab to further screen these candidate genes. The expression of candidate gene 15 which has high homology with AF1q, a newly discovered gene in leukemic cells appears to be induced by indoles in the absence of estrogen, and to be inhibited by indoles in the presence of estrogen. Studies are continuing to confirm this effect on the expression of gene AF1q and to identify further indole responsive genes.

2. Experimental methods and procedures

A. Cell Culture:

a. For Growth Experiment: MCF-7 Cells were cultured in Dulbeco's modified Eagle's medium (DMEM), with 10% fetal bovine serum, 3 gm/L glucose, 3.7 gm/L sodium bicarbonate at 370 C, 5% CO2, and 95% air. Before the treatment, cells were depleted with estrogen for at least 7 days (7-10 days) by putting them in the depletion medium composed of DMEM base without phenol-red (sigma), with 4 gm/L glucose, 3.7 gm/L sodium bicarbonate, and 5% doubly dextran-coated charcoal stripped, microfiltered calf serum (3), supplemented with non-essential amino acid (Gibco), 2 mM glutamine and 10 ng/ml insulin (1). During the depletion period, medium was changed every other day, and before adding the medium, cells were washed with ice-cold PBS once. Once the treatment starts, medium was changed every day to prevent indoles from being metabolized by the cells.

b. For pS2 gene expression: MCF-7 Cells were cultured in Dulbeco's modified Eagle's medium (DMEM), with 10% fetal bovine serum, 3 gm/L glucose, 3.7 gm/L sodium bicarbonate at 370 C, 5% CO2, and 95% air. Before the treatment, cells were depleted with estrogen for 6-7 days by putting them in the depletion medium composed of Improved modified Eagle's medium (IMEM) without phenol-red (Biofluid), with 5% dextran-coated charcoal stripped fetal bovine serum (Hyclone). During the depletion period, medium was changed every other day, and before adding the medium, cells were washed with ice-cold PBS once.

B. Transient transfection: Lipofectamine (Gibco BRL) is a mixture of polycationic and neutral lipids that bind to plasmid DNA and form a suspension of microvesicles in serum-free culture medium. After addition to culture plates the vesicles make contact with the cell membrane and facilitate the incorporation of the DNA into the cells. This method is suitable for both transient and stable transfections of eukariotic cells. A detailed protocol for use comes with the product (sold as 1.0 mL vials)
For transfection in 60 mm plates:

- It is important that the cells had been growing exponentially until that time. Do not start with confluent plates with arrested growth. Also, depleting MCF7 cells of estrogen will greatly decrease transfection efficiency.
- 24 hours before transfection seed plates with appropriate number of cells to get 50-60% confluent at the time of transfection (3 @ 70-80% confluent 100 mm plates transferred to 24 x 60 mm plates is about right for MCF7 cells.
- For one 60 mm plate (scale up for actual number of plates):
  In round bottom sterile tubes :
  Dilute Lipofectamine (from Gibco; mix gently before use) in serum free (OPTI-MEM from Gibco works very well) medium: 8µl lipid + 92 µL serum free medium.
  Dilute plasmid (i.e. 0.5 - 1.0 µg DNA for a CAT reporter) in 100 µL serum free medium.
  Combine lipid and plasmid dilutions, **mix very gently** (no vortexing or pipetting up and down, inverting the tube is okay) and let stand at room °C for 30-45 min.
- Meanwhile,wash plates with 4 mL serum free medium and feed plates with 2 mL serum free medium.
- Add 200 µL of the lipid/DNA suspension above to each plate and mix gently.
- Return plates to incubator for 5 (or 6) hours.
- Add 2 mL of medium containing 2X plasma (i.e. 10% DCC-FBS for E2 depleted conditions where cells are to be treated in 5% DCC-FBS, or 10% calf serum).
- Next day re-feed plates with fresh medium (5% DCC-FBS) and start 48 hours treatment of transients.

C. Mixed phase CAT assay (Chloramphenicol Acetyl Transferase): The mixed phase assay is a modification of the single phase extraction assay in which the hydrophobic product of the reaction -- the acylated chloramphenicol -- migrates into the organic phase constituted by the scintillation fluid. It avoids the extraction and transfer of the organic phase and allows the reaction to proceed as long as needed to obtain sufficient counts in those samples with low activity, thereby increasing sensitivity

- At the end of the treatment period (24 or 48 h), the transfected cells are harvested by scrapping with a rubber policeman, transferred with the medium to a conical 15 mL, spun at 2000 rpm for 3 min, resuspended in 1 mL cold PBS, transferred to Eppendorf tube, spun at 3000 rpm for 2 min and washed in PBS a second time. (Alternatively cells can be harvested by trypsinization, transferred to a microcentrifuge tube and centrifuged at 3000 rpm for 2 min, washed in 1 mL of PBS and centrifuged again).
- Cell pellets are resuspended in 200µL of Tris 0.1 M pH 8.0 and lysed by 3 cycles of freeze/thaw (alternate 5 min in dry-ice/alcohol and 5 min at 37°C)
- Incubate at 65°C for 15 min to inactivate potential inhibitors and centrifuge at 14000 rpm for 8 min (if using a transfection efficiency marker (ß Gal) assays take an aliquot of lysate before the 65°C treatment)
- Transfer 165 µL of cell lysate to a 7 mL scintillation vial and use (or freeze) the remainder of the lysate for protein (Bradford)

- Add 85 µL of substrate mixture** (SM) to get final concentrations of 100 mM Tris.HCl pH 8.0, 250 nmoles chloramphenicol, 1 µCi ^{3}H-acetyl CoA [200 mCi/mmol, NEN # NET-290L] and mix thoroughly.
- Add slowly (to avoid mixing with the aqueous phase where the reaction is proceeding), 4 mL of organic scintillation fluid.[EconoFluor 2 Packard # 6NE9699] Be careful not to shake the vials after this point.
- Incubate at 37°C for 1-2 hours and take a first ^{3}H count. If necessary incubate again for longer periods (up to 8 hours) to obtain counts that are clearly distinct from background for samples with very low activity

**SM: per sample 50µL chloramphenicol, 25µL Tris 1M pH7.8, 1µCi ^{3}H-acetyl CoA,total volume 85 µL Tris 0.1M

SM for **24** samples: mix in conical 15 mL on ice

chloramphenicol 5 mM (25 x 50)		1250µL
Tris 1M pH7.8 (25 x 25)		625µL
Tris 0.1M pH7.8		200µL
^{3}H-acetyl CoA 25 µCi (0.5 µCi/µL)	___	50µL
		2125µL

Add 85 µL to the scintillation vial containing 165µL cell lysate

Chloramphenicol 5mM: 81mg in 50 mL H_2O store 0-4°C

Conclusions:

We have made several potentially significant observations during this initial phase of the grant.

1) I3C inhibits or completely blocks tumor cell proliferation in estrogen dependent and independent cells. The very specific effect of I3C that we observe on estrogen independent growth suggests that this indole or its metabolites may have broader cancer protective effects than heretofore suspected. (submitted manuscript appended (15))

2) Our observation of the comparatively potent effects of indole products against cell growth is novel and, in light of recent reports on the promising cancer protective effects of this derivative in animal studies, indicates that further detailed studies of the activities of this indole are well justified.

3) Our finding that the indoles are weak antagonists of estrogen- induced reporter gene expression suggests that the antiestrogenic effects may not arise from a classical estrogen receptor antagonism as in the case of tamoxifen, for example. This finding is significant because it confirms the importance of examining effects on other estrogen responsive promoters and raises the possibility of interaction of the estrogen mediated pathway with other inhibitory receptor systems, as is the case for the antiestrogenic effects vitamin A.

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Appendix:

Submitted manuscript:

Cover, C.M., Hsieh, S. J., Tran, S. H., Kim, G. S., Bjeldanes, L. F. and Firestone, G. L. (1997) Indole-3-carbinol inhibits the expression of cyclin dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. Submitted to Journal of Biological Chemistry.

Indole-3-carbinol inhibits the expression of cyclin dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling *

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Running title: I3C inhibited CDK6 expression in breast cancer cells

ABSTRACT

Indole-3-carbinol (I3C), a naturally occurring component of *Brassica* vegetables, such as cabbage, broccoli and Brussel sprouts, has been shown to reduce the incidence of spontaneous and carcinogen-induced mammary tumors. Treatment of cultured human MCF7 breast cancer cells with I3C reversibly suppressed the incorporation of [^{3}H]thymidine without affecting cell viability or estrogen receptor (ER) responsiveness. Furthermore, flow cytometry of propidium iodide stained cells revealed that I3C induced a G1 cell cycle arrest. Concurrent with the I3C-induced growth inhibition, northern blot and western blot analyses demonstrated that I3C selectively abolished the expression of cyclin dependent kinase 6 (CDK6) in a dose and time dependent manner. After the MCF7 cells reached their maximal growth arrest, the levels of the p21 and p27 CDK inhibitors increased by 50%. The antiestrogen tamoxifen also suppressed MCF7 cell DNA synthesis, but had no effect on CDK6 expression, while a combination of I3C and tamoxifen more stringently inhibited MCF7 cell growth compared to the effects of either agent alone. The I3C-mediated cell cycle arrest and repression of CDK6 production was also observed in ER-deficient MDA-MB-231 human breast cancer cells which demonstrates that this indole can suppress the growth of mammary tumor cells independent of ER signaling. Thus, our observations have uncovered a previously undefined antiproliferative pathway for I3C that implicates CDK6 as a target for cell cycle control in human breast cancer cells. Moreover, our results establish for the first time that CDK6 gene expression can be inhibited in response to an extracellular antiproliferative signal.

INTRODUCTION

Considerable epidemiological evidence suggests that high vegetable diets correlate with low breast cancer risk (1, 2). This phenomenon is likely due to the diverse spectrum of dietary and environmental compounds that can regulate the function and proliferation of mammalian cells by influencing hormone receptor signal transduction pathways (3, 4). Several classes of these naturally occurring hormone-like chemicals have been implicated in the control of tumor cell growth and as chemopreventative agents. One such substance is the dietary compound indole-3-carbinol (I3C)[1], an autolysis product of a glucosinolate, glucobrassicin, which occurs in *Brassica* vegetables such as cabbage, broccoli, and Brussels sprouts (5, 6). A recent screen of ninety potential chemopreventative agents in a series of six short term bioassays relevant to carcinogen-induced DNA damage, oxidative stress, and tumor initiation and promotion, revealed I3C to be one of only eight compounds effective in all assays (7). Several studies have shown that exposure to dietary I3C markedly reduces the incidence of spontaneous and carcinogen-induced mammary tumors in rodents (8, 9). For example, I3C administered in the diet or by oral intubation prior to treatment with carcinogen reduced the incidence of 7,12-dimethyl-benz(α)anthracene (DMBA)-induced mammary tumors in rodents by 70-90% (6, 10). Consistent with these results, dietary supplementation with cabbage or broccoli, vegetables that are good sources of I3C, also resulted in decreased mammary tumor formation in DMBA-treated rats (11). Also, in a long-term feeding experiment in which female mice consumed synthetic diets containing I3C, spontaneous mammary tumor incidence and multiplicity were reduced by 50% and tumor latency was prolonged compared to untreated control animals **(9)**. I3C also has anticarcinogenic effects on other cancer types, such as hepatic derived tumors (12), and can reduce benzo[a]pyrene-induced neoplasia of the forestomach (6).

I3C has been shown to have an antiestrogenic activity *in vivo* which has been proposed to account for some of its protective and antiproliferative effects on mammary tumor formation. Part of this effect may be due to alterations in estrogen metabolism since oral administration of I3C to humans increased estradiol 2-hydroxylation approximately 50% in both men and women (13) and also increased the levels of estradiol hydroxylation activity in female rats (14). In addition, I3C was shown to block the estradiol-induced proliferation of long-term confluent cultures of human breast cancer cells (15). A major complication in interpreting the physiological results is that I3C is extremely unstable in acidic solution and it does not completely survive exposure to the low pH environment of the stomach (16). A relatively large fraction of I3C is converted into several acid-catalyzed derivatives with distinct biological activities that appear to mediate the antiestrogenic effects of I3C. Two of the most active acid products of I3C that have been identified are 3,3'-diindolylmethane (DIM) and indolo[3,2-*b*]carbazole (ICZ) (17). A general picture has emerged indicating that many, if not all, of the long-term antiestrogenic biological activities of I3C likely result from the actions of one or more of its acid-catalyzed derivatives (18, 17). ICZ appears to mediate its antiestrogenic effects by the direct binding to the Ah receptor (aromatic hydrocarbon or dioxin receptor), inducing P450 CYP1A1 gene expression (19) which can alter estrogen metabolism, thereby effectively decreasing the amount of circulating estrogen. This reduction in circulating estrogen leads to the decreased growth of estrogen-responsive mammary tissue and, presumably, a protective effect against breast cancer. ICZ exhibits only a very weak affinity for the estrogen receptor (ER) but is the most potent Ah receptor agonist among the characterized I3C acid-catalyzed derivative compounds. In contrast, I3C has approximately 100,000 fold lower affinity for the Ah receptor compared to ICZ (Kd of 190 pM) and 3000 fold lower affinity compared to DIM (Kd of

90 nM) (17). Thus, it is unlikely that I3C mediates its activities directly through the Ah receptor, and conceivably this dietary indole may exert many of its direct growth inhibitory effects through a signal transduction pathway distinct from the antiestrogenic effects of its acid-catalyzed products. However, virtually nothing is known about the mechanism by which I3C mediates its antiproliferative effects on human breast cancer cells. The I3C regulated cellular processes are likely to be complex because both estrogen-independent and estrogen-dependent pathways may potentially be under indole control.

Analogous to most other antiproliferative signaling molecules in mammalian cells, the I3C growth suppression pathway likely targets specific components and stages within the cell cycle. The cell cycle of eukaryotic cells is composed of four phases, G1 (gap 1), DNA synthesis, G2 (gap 2) and mitosis as well as an out of cycle quiescent phase designated G0. In normal mammary epithelial cells, an intricate network of growth inhibitory and stimulatory signals are transduced from the extracellular environment and converge on G1-acting components which, through their concerted actions, stringently regulate cell cycle progression (20, 21). The final targets of these growth signaling pathways are specific sets of cyclin-cyclin dependent kinase (CDK) protein complexes, which function at specific but overlapping stages of the cell cycle (22, 23, 24). Within the G1 phase of the cell cycle, certain cyclins (C, D1, D2, D3, E) are necessary for activation the G1 CDKs (CDK2, CDK4, and CDK6), while, in a complementary manner, several of the small proteins associated with cyclin-CDK complexes (p15, p16/Ink4a, p21/Waf1/Cip1, p27/Kip1, p57/Kip2) have been shown to act as specific inhibitors of cyclin dependent kinase activity (25, 26, 27, 28). The loss of normal cell cycle control in G1 has been implicated in mammary tumor development and proliferation. Up to 45% of human breast cancers show an aberrant expression and/or amplification

of cyclin D1 or cyclin E (29, 30). In other studies, inappropriate expression and/or mutation of certain G1-acting proto-oncogenes, growth factors and their cognate receptors have been observed in both human and rodent mammary tumor cells (31, 30, 32). Furthermore, many tumors exhibit a loss in expression or function of certain tumor-suppressor genes (such as p53) which modulate cell cycle events late in the G1 phase (33, 34).

Regulated changes in the expression and/or activity of cell cycle components that act within G1 have been closely associated with alterations in the proliferation rate of normal and transformed mammary epithelial cells (4). For example, the estrogen-induced activation of CDK4 and CDK2 during progression of human breast cancer cells between the G1 and S phases is accompanied by the increased expression of cyclin D1 and decreased association of the CDK inhibitors with the cyclin E-CDK2 complex (35). In rat tumor cells derived from DMBA-induced mammary adenocarcinomas, glucocorticoids induce a G1 cell cycle arrest and alter expression of cell cycle-regulated genes (36). Thus, it seemed likely that dietary indoles control the emergence and proliferation of breast cancer cells by regulating G1-acting cell cycle components in mammary epithelial cells. In this study, we demonstrate that the dietary indole I3C, but not the acid-catalyzed derivatives DIM or ICZ, inhibits the growth of human MCF7 breast cancer cells and induces a G1 cell cycle arrest in an ER-independent manner. Strikingly, this growth arrest is accompanied by the selective inhibition of expression of the transcripts and protein for CDK6 which acts during progression through the G1 phase of the cell cycle.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), calf serum (CS), calcium-free and magnesium-free phosphate buffered saline (PBS), L-glutamine, and trypsin-EDTA were supplied by BioWhittaker (Walkersville, MD). Insulin (Bovine), 17β-estradiol and tamoxifen ([Z]-1-[p Dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) citrate salt were obtained from Sigma Chemical Corp. (St. Louis, MO). [^{3}H]Thymidine (84 Ci/mmol) and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). I3C was purchased from Aldrich (Milwaukee, WI) and was recrystalized in hot toluene prior to use. ICZ and DIM were prepared and purified as described (17). The sources of other reagents used in the study are either listed in the following methods or were of the highest purity available.

Cell Culture. The human breast adenocarcinoma cell lines, MCF7 and MDA-MB-231, were obtained from the American *Type Culture* Collection (Rockville, MD). MCF7 cells were grown in DMEM supplemented with 10% FBS, 10 μg/ml insulin, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine. MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine. Both cell lines were maintained at 37°C in humidified air containing 5% CO_2 at subconfluency. I3C, tamoxifen and 17β-estradiol were dissolved in dimethyl sulfoxide (DMSO, 99.9% HPLC grade, Aldrich) at concentrations 1000 fold higher than the final medium concentration. In all experiments, 1 μl of the concentrated agent was added per ml of medium and for the vehicle control, 1 μl DMSO was added per ml medium.

[^{3}H]Thymidine Incorporation. Breast cancer cells were plated onto 24-well Corning tissue culture dishes. Triplicate samples of asynchronously growing

mammary cells were treated for the indicated times with either vehicle control (1 µl DMSO/ml medium) or varying concentrations of I3C, estradiol, and/or tamoxifen. The cells were pulsed for three hours with 3 µCi [^{3}H]thymidine (84 Ci/mmol), washed three times with ice cold 10% trichloroacetic acid, and lysed with 300 µl 0.3N NaOH. Lysates (150 µl) were transferred into vials containing liquid scintillation cocktail and radioactivity was quantitated by scintillation counting. Triplicates were averaged and expressed as counts per minute per well.

Flow Cytometric Analyses of DNA Content. Breast cancer cells (4 x 10^4) were plated onto Corning 6-well tissue culture dishes. A final concentration of 100 µM I3C was added to three of the wells and vehicle control (1 µl DMSO/ml medium) was added to the other three. The medium was changed every 24 hours. Cells were incubated for 96 hours and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). Nuclear emitted fluorescence with wavelength greater than 585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Nuclei (10,000) were analyzed from each sample at a rate of 300-500 cells/second. The percentages of cells within the G1, S, and G2/M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

Western Blot Analysis. After the indicated treatments, cells were harvested in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% NP-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 µg/ml PMSF, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 0.1 µg/ml NaF, 10 µg/ml β-glycerophosphate). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol,

0.075% SDS, 1.25 ml 14.4 M 2-mercaptoethanol, 10% bromophenol blue, 3.13% stacking gel buffer) and fractionated on 10% polyacrylamide/0.1% SDS resolving gels by electrophoresis. Rainbow marker (Amersham Life Sciences, Arlington Heights, IL) was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) and blocked overnight at 4°C with western wash buffer/5% NFDM (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20/5% nonfat dry milk). Blots were subsequently incubated for 1 hour at room temperature for rabbit anti-CDK2, CDK4, CDK6 and ER antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA Cat. #sc-163, sc-260, sc-177, sc-543 respectively), 2 hours at room temperature for goat anti-p21, rabbit anti-cyclin E, and rabbit anti-p27 antibodies (Santa Cruz Biotechnology, Inc. Cat. #sc-397, sc-198, sc-528 respectively) and overnight at 4°C for mouse anti-cyclin D1 (Santa Cruz Biotechnology, Inc. Cat. #sc-246). Working concentration for all antibodies was 1 µg/ml western wash buffer. Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibody diluted to 3 x 10^{-4} in western wash buffer/1% NFDM (goat anti-rabbit IgG, BioRad, Hercules, CA, rabbit anti-mouse IgG, Zymed, SF, CA, donkey anti-goat IgG, Santa Cruz Biotechnology, Inc.). Blots were treated with ECL reagents (NEN Life Science Products) and all proteins were detected by autoradiography. Equal protein loading was confirmed by Ponceau S staining of blotted membranes.

Quantitation of Autoradiography. Autoradiographic exposures were scanned with a UMAX UC630 scanner and band intensities were quantified using the NIH Image program. Autoradiographs from a minimum of three independent experiments were scanned per time point.

Isolation of Poly(A)+ RNA and Northern Blot Analysis. Poly(A)+RNA was isolated from MCF7 cells treated with either 100 μM I3C or vehicle control (1 μl DMSO/ml medium) for 5, 15, or 24 hours as previously described (37). For northern blot analysis, 10 μg of poly(A)+RNA was electrophoretically fractionated in 6% formaldehyde/1% agarose gels, transferred onto Nytran nylon membranes (Schleicher and Schuell, Keene, NH) and UV crosslinked in a UV Stratalinker (Stratagene, La Jolla, CA). The RNA ladder 0.24-9.5 Kb (Gibco BRL, Gainthersburg, MD) was used as the molecular weight standard. Membranes were preannealed with 100 μg/ml denatured salmon sperm DNA in hybridization buffer (5X Denhardt's reagent, 5X SSC, 50% formamide) and subsequently hybridized for 16-20 hours with cDNA probes [α^{32}P]dCTP-labeled by random primer extension (Boehringer Mannheim, Indianapolis, IN). For detection of CDK6 transcripts, the cDNA probe was added to the hybridization buffer at a concentration of six million cpm/ml. CDK6 membranes were washed twice for 10 min with 2X SSC at room temperature followed by two hour-long washes with 0.2X SSC/0.1%SDS at 60°C. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CDK2, and CDK4 transcripts, the cDNA probe was added to the hybridization buffer at a concentration of two million cpm/ml. These membranes were subsequently washed with 2X SSC twice at room temperature for 10 min followed by two 30 min washes at 60°C in 0.2 X SSC/ 0.1% SDS. The membranes were placed under film and analyzed after 4 hour-2 day exposures at -80°C. CDK2, 4, and 6 transcripts were detected using purified *Bam*H1 fragments of the pCMVcdk2, pCMVcdk4, or pCMVcdk6 plasmids containing the respective cDNA for each of the CDKs (the CDK plasmids were a generous gift from Ed Harlow's laboratory as described (38)). GAPDH transcripts were detected with a 560 bp *Xba*I/*Hin*D III cDNA fragment of the corresponding cDNA.

Transfection Procedure. Subconfluent breast cancer cells were propagated for at least one week in either low (5% CS) or high serum (10% FBS) containing medium prior to plating onto 60-mm Corning dishes 24 hours before transfection in the appropriate medium. DNA-lipofectamine (Gibco BRL) mixtures were prepared by mixing 1 µg of ERE-vit-CAT reporter (-596 to +21 of the *Xenopus laevis* vitellogenin B1 genomic clone plus a consensus estrogen response element (ERE) subcloned into the *Hind*III site upstream of the chloramphenicol acetyl transferase (CAT) reporter gene in the SVOCAT vector, a gift from D. J. Shapiro, Univ. of Illinois) with 5 µl lipofectamine in a total volume of 100 µl for 15 minutes at room temperature. The cells were washed twice with serum-free medium and 100 µl of the liposome complex was added to each plate. After a five hour incubation at 37°C, the transfection was terminated by adding an equal volume of 2X media containing either 10% CS or 20% FBS. Complete medium was replaced 18-24 hours post-transfection, at which time appropriate agents (eg. 17β-estradiol, tamoxifen, I3C) were added. pCAT-basic vector (Promega, Madison, WI), which contains the promoterless CAT cDNA, was used as a negative control to determine background CAT activity.

CAT Assay. Cells were harvested by washing in PBS, resuspended in 100 mM Tris HCl pH 7.8, and lysed by three freeze/thaw cycles, 5 minutes per cycle. Cell lysates were heated at 68°C for 15 minutes, centrifuged at $1.4 \times 10^4 \times g$ for 10 minutes and the supernatant fractions were recovered. CAT activity in the cell extracts containing 20-50 µg of lysate protein was measured by a quantitative nonchromatographic assay (39). The enzyme assay was carried out in 100 mM Tris HCl pH 7.8, 1 mM aqueous chloramphenicol, and 1 µCi [^{3}H]acetyl coenzyme A (final reaction volume of 250 µl). The reaction mixture was overlaid with 4 ml of

Econofluor scintillation fluorochrome (NEN Life Science Products). CAT activity was monitored by direct measurement of radioactivity by liquid scintillation counting. Measurements of CAT activity were in the linear range of the assay as determined by a standard curve using bacterial CAT enzyme (0.01 units; Pharmacia, Uppasala, Sweden), the positive control for CAT enzymatic activity. Reaction mixtures were incubated at 37°C for 2-6 hours. Mock transfected cells were used to establish basal level activity for both assays. The enzyme activity was expressed as CAT activity produced per μg protein present in corresponding cell lysates, as measured by Bradford assay, and the results show averages of triplicate samples.

5-Bromo-2'-deoxyuridine (BrdU) Incorporation and Indirect Immunofluorescence. MCF7 cells grown on 8-well Lab-Tek Permanox slides (Nalge Nunc International, Naperville, IL) were treated for 96 hours with either vehicle control (1 μl DMSO/ml media) or 100 μM I3C followed by incubation with fresh medium containing a final concentration of 100 μM BrdU (Sigma, St. Louis, MO) at 37°C for 2 hours. Cells were washed with PBS, fixed for 30 minutes in 4% paraformaldehyde, rinsed with PBS and DNA was denatured by incubation in 0.12 N HCl at 37°C for 1 hour. After neutralization in two changes of 0.1 M borate buffer over 10 minutes, cells were washed in PBS and blocked for 5 minutes in PBS containing 4% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were then incubated with mouse monoclonal anti-BrdU antibody (DAKO Corporation, Carpinteria, CA; diluted 1:80 in PBS) for 60 minutes at 25°C. After five washes with PBS, cells were blocked for 5 minutes in PBS containing 4% normal goat serum. Cells were then incubated in anti-mouse rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; diluted 1:300 in PBS) at 4°C for 30 minutes. Finally, cells were washed five times with PBS, mounted with 50% glycerol/50 mM Tris (pH 8.0), and

examined with a Nikon Optiphot fluorescence microscope. Images were captured using Adobe Photoshop 3.0.5 and a Sony DKC-5000 digital camera. Nonspecific fluorescence, as determined by incubation with secondary antibody alone, was negligible.

RESULTS

I3C reversibly inhibits the growth of human MCF7 breast cancer cells. As an initial test to determine whether dietary indoles can directly regulate the growth of human breast cancer cells, MCF7 cells were cultured at subconfluency in medium supplemented with 10% FBS and 10 μg/ml insulin and then treated with several concentrations of I3C for 48 hours. Cells were then pulse-labeled with [^{3}H]thymidine for three hours to provide a measure of the proliferative state of the cells. Analysis of [^{3}H]thymidine incorporation revealed a strong dose-dependent inhibition of DNA synthesis with half-maximal response at approximately 30 μM I3C (Figure 1, upper panel). Treatment with I3C above 200 μM was toxic to the cells. The lowest concentration of I3C that maximally inhibited the growth of MCF7 cells without affecting viability was 100 μM, and this level of the indole was therefore routinely used in subsequent experiments. *In vivo*, I3C is converted into several acid-catalyzed products, including DIM and ICZ, which bind to and activate the Ah receptor. Neither of these acid-catalyzed products had any significant effect on the incorporation of [^{3}H]thymidine into MCF7 breast cancer cells after 48 hour treatments with concentrations that were above the Kd for the Ah receptor (data not shown). In multiple experiments, ICZ and DIM caused only an average of 10% inhibition of DNA synthesis under these conditions, which was not statistically significant.

Time course studies of I3C addition and withdrawal demonstrated that the I3C growth suppression of MCF7 breast cancer cells is completely reversible, therefore this compound does not affect cell viability. For example, analysis of DNA synthesis over a 96 hour time course revealed that 100 μM I3C inhibited [^{3}H]thymidine incorporation by 80% after 72 hours and by greater than 90% after 96

hours of treatment (Fig. 1, lower panel). The rate of [^{3}H]thymidine incorporation in untreated cells was approximately equivalent to the rate observed 48 hours after indole withdrawal (Fig. 1, lower panel). Even after prolonged exposure (50 days), the I3C-induced growth arrest was readily reversible (data not shown).

I3C induces a G1 cell cycle arrest of MCF7 breast cancer cells and rapidly abolishes the production of the CDK6 cell cycle component. To assess the cell cycle effects of I3C, MCF7 cells were treated with or without 100 μM I3C for 96 hours and were then hypotonically lysed in the presence of propidium iodide to stain the nuclear DNA. Flow cytometry profiles of nuclear DNA content revealed that I3C induced a cell cycle arrest of these breast cancer cells. As shown in Figure 2, I3C treatment altered the DNA content of the MCF7 cell population from an asynchronous population of growing cells (Fig. 2, upper panel) in all phases of the cell cycle (26% in G1, 52% in S phase, and 22% in G2/M phase) to one in which most (73%) of the I3C-treated breast cancer cells exhibited a 2n DNA content (Fig. 2, lower panel), which is indicative of a G1 block in cell cycle progression. These results suggest that I3C suppresses cell growth by inducing a specific block in cell cycle progression.

To determine potential downstream targets of the I3C-activated pathway that induces the cell cycle arrest, the expression of components that function within the G1 phase of the cell cycle was examined during a time course of I3C treatment. MCF7 cells were treated with or without 100 μM I3C for the indicated time periods (Fig. 3) and the production of G1 CDKs, cyclins, and CDK inhibitors was examined by western blot analysis. Among the array of cell cycle components tested, only CDK6 protein levels were rapidly and significantly reduced in response to I3C treatment.

The level of CDK6 is reduced within 24 hours of indole treatment, and by 96 hours CDK6 production is essentially abolished (Fig. 3, upper panel). Importantly, no effect was observed on the expression of the two other G1-acting cyclin dependent kinases, CDK2 or CDK4. This result demonstrates the specificity of the I3C response. In addition, I3C did not alter the level of either cyclin D1 or cyclin E, which have been shown to be regulated in other studies. Estrogens and progesterone stimulate and antiestrogens inhibit cell cycle progression of the T47D human breast cancer cell line at a point in early G1 phase of the cell cycle with corresponding changes in cyclin D1 and p21 expression (40, 41). Quantitative analysis of autoradiographs from more detailed time courses (Fig. 3, lower panel) revealed that of all the cell cycle components, the level of CDK6 protein was the only one which correlated approximately with the inhibition of DNA synthesis (see Fig. 1), suggesting a causal relationship between these two effects of I3C. Western blot analysis also demonstrated that I3C gradually stimulated the levels of the p21 and p27 cell cycle inhibitors by 50% only after the cells begin to display their maximal cell cycle arrest.

I3C inhibition of CDK6 transcript expression. Because the level of CDK6 protein is rapidly reduced by indole treatment, it seemed likely that I3C regulates the level of CDK6 transcripts. Poly(A)+RNA was isolated from MCF7 breast cancer cells treated with or without 100 μM I3C for the indicated times and electrophoretically fractionated samples were examined by northern blot analysis. As shown in Figure 4 (upper left panel), 15 hours treatment with I3C caused a specific reduction in the level of both the 6 kb and 13 kb CDK6 transcripts. By 24 hours of I3C treatment, the expression of CDK6 transcripts was undetectable (Fig. 4, upper right panel). Consistent with the unaltered protein production observed for CDK2 and CDK4, the transcript levels for these CDKs remained unchanged in either the presence or absence of I3C for 24 hours (Fig 4, upper right panel). It is worth mentioning that

MCF7 cells produce a significantly lower amount of CDK6 mRNA compared to the level of either CDK2 or CDK4 transcripts. A two-day X-ray film exposure is required to detect the CDK6 mRNA bands with 10 μg Poly(A)+ sample, whereas the same blot reprobed for CDK2 or CDK4 can be developed within 6 hours. Autoradiographs from multiple experiments were quantitated and CDK6 mRNA concentrations at each time point were normalized to the level of GAPDH transcripts, a constitutively expressed gene. No significant effect of I3C on CDK6 transcript levels were observed after 5 hours treatment. By 15 hours, I3C caused a 5-fold reduction in CDK6 mRNA levels and within 24 hours of indole treatment, CDK6 transcripts were almost undetectable (Fig. 4, lower panel). These changes in CDK6 transcript levels appear to account for the reduction in CDK6 protein, because CDK6 protein levels begin to decrease in response to I3C between 15 and 21 hours of treatment (Fig. 3, lower panel). Taken together, our results demonstrate that under conditions in which I3C induces a cell cycle arrest of MCF7 breast cancer cells, the expression of both transcripts and protein for CDK6, a G1-acting cell cycle component, is selectively reduced.

The regulation of CDK6 production is specific for the antiproliferative effects of I3C. An important question is whether the I3C-induced reduction in CDK6 expression is a specific I3C response or whether it is a general consequence of the inhibition of cell growth. To distinguish these possibilities, the effects of I3C were compared to that of the antiestrogen, tamoxifen, a well known inhibitor of the proliferation of estrogen-treated MCF7 cells (42). The I3C treatments were performed using MCF7 cells cultured in medium supplemented with 10% FBS which contains enough estrogen and growth factors to maintain the cells in a proliferative state. To first demonstrate that tamoxifen can selectively inhibit an estrogen responsive reporter plasmid in the presence of 10% FBS, ER expressing

MCF7 breast cancer cells were transiently transfected with the ERE-vit-CAT reporter plasmid which contains the vitellogenin promoter with four estrogen response elements linked upstream and driving the bacterial CAT gene. The cells were treated for 48 hours with the indicated combinations of estrogen, tamoxifen and/or I3C (Fig. 5) and the reporter gene activity was assayed by monitoring the conversion of [^{3}H]acetyl-CoA and unlabeled chloramphenicol into [^{3}H]acetylchloramphenicol. As shown in Figure 5, the medium used for I3C-induced growth suppression (10% FBS) has endogenous estrogen and growth factors at a sufficient concentration to cause a high basal level of reporter gene activity in MCF7 cells transiently transfected with the ERE-vit-CAT reporter plasmid compared to the low serum (5% CS) condition. Low serum medium, as opposed to serum-free medium, was chosen because MCF7 cells cultured in serum-free conditions were not transfection competent. Treatment with 100 nM 17β-estradiol further stimulated ERE-vit-CAT activity above each of the basal serum levels. In contrast, tamoxifen inhibited the serum-induced ERE-vit-CAT reporter plasmid activity by 70%. Treatment with I3C had no effect on the ER responsiveness of the ERE-vit-CAT activity nor did this dietary indole modulate the antagonistic effects of tamoxifen (Fig. 5). Thus, under the FBS-containing conditions utilized to culture MCF7 cells, tamoxifen acts as a potent antagonist of ER responsiveness, while I3C has no apparent effects on ER function.

To test whether I3C and tamoxifen affected the absolute number of S phase cells, MCF7 cells were treated for 48 hours with 100 μM I3C and/or 1 μM tamoxifen, and the cells exposed to a 2 hour pulse of BrdU which was used as a measure of DNA synthesis on a single cell level. The incorporation of BrdU was monitored by indirect immunofluorescence using BrdU-specific antibodies. Representative photographs are shown in Figure 6. The number of BrdU-incorporating cells was

quantitated by examining approximately 500-1000 cells per condition. In an asynchronous growing population, 21.5 % of MCF7 cells incorporated BrdU. Treatment with either I3C or with tamoxifen suppressed BrdU incorporation to 4.7% and 12.3% respectively. Treatment with both reagents induced a more effective growth suppression (3.8%) compared to either I3C or tamoxifen alone. Treatment with I3C or tamoxifen caused the cells to show a more flattened morphology which is consistent with the effects of other antiproliferative agents (43, 44).

Because I3C and tamoxifen both inhibited cell growth, the effects of these two antiproliferative agents on CDK6 production were examined. MCF7 cells were treated with combinations of I3C and tamoxifen over a 96 hour time course and cell extracts were analyzed for CDK protein by western blots. I3C, but not tamoxifen, reduced the level of CDK6 protein and no effects were observed on CDK4 or CDK2 production with either reagent (Fig. 7). A combination of I3C and tamoxifen reduced CDK6 protein levels to approximately the same extent as I3C alone. These results indicate that the inhibition of CDK6 expression is a specific I3C response and not a general consequence of growth arrest in MCF7 cells. Because the effects of the antiestrogen tamoxifen and I3C differ, these results suggest that I3C may act, in part, through an ER-independent pathway to suppress breast cancer cell growth.

I3C can suppress the growth and reduce CDK6 production in an ER deficient human breast cancer cell line The ER-deficient MDA-MB-231 cells were utilized to demonstrate that I3C can suppress the growth of human breast cancer cells independent of any effects on ER responsiveness. Western blot analysis using antibodies to the human ER confirmed that the MDA-MB-231 breast cancer cells do not express an ER protein, while MCF7 cells produce the ER (Fig. 8, upper panel). Moreover, consistent with the lack of ER protein, neither estrogen nor tamoxifen

had any effects on ERE-vit-CAT reporter activity in transiently transfected MDA-MB-231 cells (data not shown). To test whether I3C can suppress the growth of human breast cancer cells in an ER-independent manner, MCF7 cells which contain ERs and MDA-MB-231 cells which do not contain ERs were treated with combinations of I3C and tamoxifen for 48 hours and DNA synthesis assayed as a measure of the incorporation of [^{3}H]thymidine. As shown in Figure 8, middle panel, treatment with either I3C or tamoxifen inhibited MCF7 DNA synthesis by approximately 70% and 60%, respectively, compared to vehicle controls. Consistent with a more stringent growth inhibitory effect of both reagents, a combination of both I3C and tamoxifen inhibited [^{3}H]thymidine incorporation by greater than 90%. In contrast, I3C, but not tamoxifen, strongly inhibited [^{3}H]thymidine incorporation in the ER deficient MDA-MB-231 cells (Fig. 8, lower panel). A combination of I3C and tamoxifen suppressed DNA synthesis to approximately the same extent as I3C alone. This result demonstrates that I3C can suppress breast cancer cell growth independent of ER-mediated events.

The ER-positive MCF7 cells and ER-negative MDA-MB-231 cells were utilized to examine the relationship between the estrogen-independent suppression of cell growth by I3C and the reduction in CDK6 protein. Cells were treated with increasing concentrations of I3C for 48 hours and the level of G1-acting CDK proteins were analyzed by western blots. As shown in Figure 9, upper panels, this indole can dose dependently reduce CDK6 production in both cell lines while in the same extracts, CDK4 and CDK2 protein levels remained unaffected. Quantitative analysis of the western blots and a parallel analysis of [^{3}H]thymidine incorporation of the I3C dose response revealed that the inhibition of CDK6 protein levels approximately correlated with the I3C-mediated decrease in DNA synthesis. Consistent with the MDA-MB-231 cells not being as stringently growth suppressed, I3C reduces CDK6

levels to a lesser extent compared to the MCF7 cells. Thus, I3C can suppress the growth and reduce CDK6 production in an ER-independent manner.

DISCUSSION

Extracellular regulators of cell proliferation, such as steroid and protein hormones, can transduce an intricate network of growth inhibitory and stimulatory signals that converge on specific sets of cell cycle components, which through their concerted actions, either drive cells through critical cell cycle transitions or inhibit cell cycle progression (21, 3). Dietary and environmental compounds that alter cell growth are likely to mediate many of their effects through signal transduction pathways analogous to the known mechanisms of hormone receptor signaling. Our results demonstrate the existence of a distinct growth inhibitory pathway that establishes a direct link between the regulation of cell cycle control by the dietary indole I3C and the selective control of cell cycle components. The unique feature of this response is that the I3C-mediated growth arrest is accompanied by the specific inhibition of expression of CDK6 transcripts and protein. The selective regulation of CDK6 expression in mammary epithelial cells by I3C provides the basis to propose a biological mechanism by which I3C potentially controls the emergence and proliferation of breast cancer cells. In this regard, several recent studies have demonstrated that CDK6 expression and activity is altered in a manner which correlates with the transformed state. For example, tumor-specific amplification of CDK6 has recently been observed in human gliomas (45), and the activity of CDK6 is amplified in certain human squamous cell carcinoma lines (46).

Changes in cyclin or CDK inhibitor expression are thought to be the key regulatory mechanisms controlling CDK function while, except for a few cases, expression of the G1-acting CDKs generally remain constant under conditions that either inhibit or stimulate cell cycle progression (23, 47, 48). For example, staurosporine treatment of human breast cancer cells causes a minor reduction in

CDK6 protein levels that accompanies a G1 cell cycle arrest (49). Treatment of T-cells with the herbimycin A tyrosine kinase inhibitor reduces the stability of the CDK6 protein (50) while both CDK4 and CDK6 protein production was suppressed by the apopototic signaling through the sIgM surface antigen receptor in B-cells (51). CDK6 functions during progression through the G1 phase of the cell cycle (52). However, relatively little is known about the influence on CDK6 expression by regulators of cell growth. In our studies with human MCF7 breast cancer cells, both time course and dose response assays with I3C revealed that the loss in CDK6 production closely coincided with the reduction in cell proliferation which implicates CDK6 as a direct target for cell cycle control in human breast cancer cells. The rapid I3C effect on CDK6 gene expression is unique because it is the first report of a growth inhibitor decreasing the mRNA for a CDK. Within fifteen hours of I3C treatment, there is a significant reduction in CDK6 transcript levels which likely accounts for the decrease in CDK6 protein. In contrast, no obvious changes were observed in the production of the other G1-acting CDKs and cyclins. Thus, the selective I3C-mediated loss in production of the CDK6 protein appears to be an important early event in the G1 cell cycle arrest of human breast cancer cell lines.

Our results have also established that I3C signaling can induce the G1 cell cycle arrest of cultured human MCF7 breast cancer cells in an estrogen-independent manner. Most strikingly, the I3C-mediated cell cycle arrest and repression of CDK6 production was observed in ER deficient MDA-MB-231 human breast cancer cells under conditions in which the antiestrogen tamoxifen had no effect on cell growth. In ER containing MCF7 cells, tamoxifen suppressed DNA synthesis to approximately the same extent as I3C, but had no effect on CDK6 expression. Moreover, while tamoxifen reduced the activity of an ERE-containing reporter plasmid in cells

cultured with FBS containing endogenous estrogens, I3C had no effect on ER responsiveness under these same culture conditions. Consistent with I3C and tamoxifen acting through distinct antiproliferative pathways, a combination of I3C and tamoxifen inhibited MCF7 cell growth to a greater extent compared to the effects of either agent alone.

Tamoxifen has been a clinically useful antiestrogen (53, 54, 55), however, 60-75% of patients with metastatic breast cancer have ER-positive tumors and only approximately half of those patients will respond to tamoxifen therapy. Therefore only 35% of metastatic breast cancer patients actually benefit from tamoxifen therapy (56). All patients who initially respond to therapy will eventually develop acquired tamoxifen resistance following prolonged administration. The cellular and molecular mechanisms underlying the development of acquired resistance to antiestrogens is unclear, but it has been proposed that continued exposure of cells to tamoxifen may select for hormone-independent, resistant cells (57). Our results suggest that I3C, in combination with tamoxifen, could prove to be an effective therapy. Patients could receive intermittent pulses of tamoxifen or lower doses of tamoxifen, both of which are proposed methods of circumventing tamoxifen resistance, while on I3C treatment. I3C has been shown to reduce the formation of both spontaneous and carcinogen-induced mammary tumors in rodents with no apparent side-effects (58, 59, 60, 9) and human subjects treated with I3C also had no side-effects (13, 61).

Taken together with previous studies on the acid-catalyzed products of I3C (62, 18), our results with I3C indicate that dietary indoles are likely to work through both ER-independent and ER-dependent pathways (see illustration in Figure 10). It is tempting to consider that I3C, or perhaps a cellular metabolite of I3C, interacts

with a putative indole receptor to reduce the expression and activity of the CDK6 cell cycle component resulting in the G1 arrest of breast cancer cells by an ER-independent pathway. Conceivably, the decrease in CDK6 protein could functionally be involved in the I3C mediated cell cycle arrest by either directly decreasing the phosphorylation of the retinoblastoma (Rb) protein or by releasing bound inhibitors which could then decrease the activity of CDK2 and/or CDK4, even though the expression of these CDKs remain constant. Consistent with this idea, a 50% increased expression of the p21 and p27 CDK inhibitors is observed after the cells reached their maximal cell cycle arrest. This increased production of the CDK inhibitors appears to be enough to reduce the Rb protein phosphorylation activities of the G1-acting CDK-containing protein complexes.[2]

The ER-dependent pathway presumably involves the activation of the Ah receptor by the acid-catalyzed products of I3C and subsequent regulation of estrogen metabolism (17). In our studies, no effects on growth were observed within 48 hours of treatment with ICZ or DIM. However, it is likely that the ER-dependent effects of these indoles require treatment periods of longer than a week (9). The regulation of CDK6 expression appears to be specific for the I3C-mediated ER-independent pathways. Several studies with human breast cancer cells have shown that the ER pathway stimulates cell proliferation by targeting the expression and/or activity of G1-acting cell cycle components (63, 3, 48). Although the immediate promoter targets of the ER are unknown, in MCF7 cells the estrogen-induced activation of CDK4 and CDK2 kinase activity was shown to be accompanied by the increase in cyclin D1 expression and decreased CDK inhibitor association with the cyclin E-CDK2 protein complex (35). No changes in CDK6 expression were reported in these studies, although in other human breast cancer cells, antiprogestin or antiestrogen treatment was shown to increase production of p21 (41). Thus, one of

our future goals will be to identify the putative indole receptor and to characterize the ER-independent pathway by which I3C regulates CDK6 expression and cell cycle control in human breast cancer cells.

Acknowledgments. We express our appreciation to Dixie Ching, Erin J. Cram, Gunnel Hallden and Anita C. Maiyar for their critical evaluation of this manuscript and helpful experimental suggestions. We also thank other members of both the Firestone and Bjeldanes laboratories for their helpful comments throughout the duration of this work. We wish to thank Wei-Ming Kao, Peter Schow, Khanh Tong, Vinh Trinh and Linda Yu for their technical assistance. We are also grateful to Jerry Kapler for his excellent photography and Anna Fung for her help in the preparation of this manuscript.

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FOOTNOTES

*This work was supported by a grant from the Department of Defense Army Breast Cancer Research Program awarded to LJB and by a National Institutes of Health grant awarded to GLF.

‡ Predoctoral trainee supported by a National Research Service Grant (CA-09041) awarded by the NIH.

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[1] The abbreviations used are: I3C, indole-3-carbinol; Ah, aromatic hydrocarbon; BrdU, 5-bromo-2'-deoxyuridine; CAT, chloramphenicol acetyl transferase; CDK, cyclin dependent kinase; CKI, cyclin dependent kinase inhibitor; CS, calf serum; DIM, 3,3'-diindolylmethane; DMBA, 7,12-dimethyl-benz(a)anthracene; DMEM, Dulbecco's Modified Eagle medium; DMSO, dimethyl sulfoxide; ER, estrogen receptor; ERE, estrogen response element; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICZ, indolo[3,2-*b*]carbazole; NFDM, nonfat dry milk; PBS, phosphate buffered saline; Rb, retinoblastoma.

[2] C. M. Cover and G. L. Firestone, unpublished results

FIGURE LEGENDS

Fig. 1. **Effects of I3C on DNA synthesis in MCF7 breast cancer cells.** Top Panel: MCF7 cells were plated at 20,000 cells per well on 24-well tissue culture dishes (forming a subconfluent monolayer) and treated with the indicated concentrations of I3C (see structure) for 48 hours. Cells were labeled with [^{3}H]thymidine for 3 hours and the incorporation into DNA was determined by acid precipitation as described in the Experimental Procedures. The reported values are an average of triplicate samples. Lower Panel: MCF7 cells were treated with 100 μM I3C (+I3C, ■) or with the DMSO vehicle control (vehicle control, ○) for a 96 hour time course. After 48 hours of I3C treatment, the I3C-containing medium in a subset of the cell cultures was replaced with medium containing only the vehicle control (-I3C, ❑) and the time course continued for an additional 48 hours. At the indicated time points for each condition, the cells were labeled with [^{3}H]thymidine for 3 hours and the incorporation into DNA determined by acid precipitation. The reported values are an average of triplicate samples.

Fig. 2. **Effects of I3C on cell cycle phase distribution of MCF7 breast cancer cells.** MCF7 cells were treated with 100 μM I3C or with the vehicle control (DMSO) for 96 hours. Cells were then stained with propidium iodide and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite Laser. A total of 10,000 nuclei were analyzed from each sample. The percentages of cells within the G1, S, and G2/M phases of the cell cycle were determined as described in the Experimental Procedures.

Fig. 3. **Effects of I3C on expression of G1 cell cycle proteins in MCF7 breast cancer cells.** Upper Panel: MCF7 cells were treated with 100 μM I3C or with the vehicle control (DMSO) for the indicated times and the protein production of the G1 cell cycle components was determined by western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each cell cycle protein and equal sample loading was confirmed by Ponceau S staining of the western blot membrane. Lower Panel: The relative level of each cell cycle component shown in the representative western blot in the upper panel, as well as from other western blots with additional time points, were quantitated as described in the Experimental Procedures. The percent of growing control was calculated by dividing the densitometry measurements of I3C-treated cells by the densitometry measurements of vehicle control-treated growing cells for each assay (CDK6 ○, p21 Δ, p27 ❑, CDK2, CDK4, cyclin D1, and cyclin E ■).

Fig. 4. **Effects of I3C on the expression of G1 CDK transcripts in MCF7 breast cancer cells.** Upper Panels: Poly(A)+ RNA isolated from MCF7 cells treated with or without 100 μM I3C for 5, 15 and 24 hours. The 5 and 15 hour treated RNAs were electrophoretically fractionated and northern blots were probed for CDK6 transcripts as described in the Experimental Procedures (left panel). The 24 hour poly(A)+RNA samples were probed for CDK6, CDK4, and CDK2 (right panel). As a loading control, the CDK6 northern blots were reprobed for GAPDH which is a constitutively expressed transcript. The X-ray film exposure times were two days for CDK6, six hours for CDK2 and two hours for CDK4 and GAPDH. Molecular weight standards in kilobases are indicated to the left of the left panel. Lower Panel: The band intensities of CDK6 and GAPDH transcripts for each condition were quantitated as described in the Experimental Procedures. CDK6 mRNA levels were normalized to

GAPDH mRNA levels by dividing the band intensities of CDK6 by the band intensity for GAPDH. The reported values represent an average of three independent experiments.

Fig. 5. **Effects of I3C and tamoxifen on the ERE-vit-CAT reporter plasmid activity in MCF7 breast cancer cells.** MCF7 cells were first cultured for one week in either 5% calf serum (CS) or 10% fetal bovine serum (FBS). Cells were then transfected with the ERE-vit-CAT reporter plasmid which encodes four estrogen response elements (ERE) within the vitellogenin promoter linked upstream to and driving the bacterial chloramphenicol acetyl transferase (CAT) gene. Transfected cells were treated with the indicated combinations of 100 nM 17β-estradiol (E2), 100 μM I3C and/or 10 μM tamoxifen (Tam) or with a vehicle control (none) for 48 hours and assayed for CAT activity by a quantitative method which measures the conversion of [^{3}H]acetyl coenzyme A into [^{3}H]acetylchloramphenicol. CAT specific activity is the CAT activity produced per μg protein present in the corresponding cell lysates. The reported values are an average of four independent experiments of triplicate samples.

Fig. 6. **Single cell analysis of the effects of I3C and tamoxifen on DNA synthesis in MCF7 breast cancer cells.** MCF7 cells cultured on 8-well slides were treated with the indicated combinations of 100 μM I3C and/or 1 μM tamoxifen (Tam) or with only DMSO (vehicle control) for 96 hours. Cells were then labeled with 100 μM bromo-2'-deoxyuridine (BrdU) for 2 hours, fixed in paraformaldehyde and incubated with mouse anti-BrdU antibodies. BrdU incorporating cells were

then visualized by fluorescence microscopy using anti-mouse rhodamine-conjugated secondary antibodies as described in the Experimental Procedures.

Fig. 7. **Effects of I3C and tamoxifen on the expression of G1 CDK proteins in MCF7 breast cancer cells.** MCF7 cells were treated with combinations of 100 μM I3C and/or 1 μM tamoxifen or with the vehicle control (DMSO) for the indicated times. The protein production of CDK6, CDK4 and CDK2 was determined by western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each CDK protein and equal sample loading was confirmed by Ponceau S staining of the western blot membrane.

Fig. 8. **Effects of I3C and tamoxifen on DNA synthesis in ER-containing and ER-deficient breast cancer cells.** Upper Panel: Expression of estrogen receptor (ER) was confirmed in ER-containing MCF7 cells and ER-deficient MDA-MB-231 (MDA) cells. ER protein expression was determined by western blot analysis using specific antibodies and equal sample loading was confirmed by Ponceau S staining of the western blot membrane. Lower Panels: MCF7 and MDA cells were plated at 20,000 cells per well on 24-well tissue culture dishes and treated with the indicated combinations of 100 μM I3C and/or 1 μM tamoxifen or with the vehicle control (DMSO) for 48 hours. Cells were labeled with [^{3}H]thymidine for 3 hours and the incorporation into DNA was determined by acid precipitation as described in the Experimental Procedures. The reported values are an average of triplicate samples.

Fig. 9. **Dose response effects of I3C on the expression of G1 CDK proteins and DNA synthesis in ER-containing and ER-deficient breast cancer cells.** Upper Panels: ER-containing MCF7 (upper left) and ER-deficient MDA-MB-231 (upper right) cells were treated with the indicated concentrations of I3C for 48 hours. The protein production of CDK6 (O), CDK4 (Δ) and CDK2 (❑) was determined by western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each CDK protein and equal sample loading was confirmed by Ponceau S staining of the western blot membrane. Lower Panels: The relative level of each CDK shown in the representative western blots in the upper panels, as well as from other western blots, were quantitated as described in the Experimental Procedures. The reported values were calculated as the percentage of vehicle control-treated growing cells (0 μM I3C) by dividing the densitometry measurements of I3C-treated cells by the measurements of vehicle control cells for each assay. In parallel with the western blots, triplicate sets of MCF7 and MDA-MB-231 cells were plated at 20,000 cells per well on 24-well tissue culture plates and treated with the indicated concentrations of I3C for 48 hours. Cells were labeled with [^{3}H]thymidine for 3 hours and the incorporation into DNA was determined by acid precipitation as described in the Experimental Procedures ([^{3}H]Thy ■).

Fig. 10. **Model for the ER-independent and ER-dependent anti-proliferative effects of I3C in breast cancer cells.** For the ER-independent pathway, I3C suppresses breast cancer cell growth by a rapid inhibition of CDK6 expression and a later stimulation of CDK inhibitor (CKI) production. We propose that this effect causes an inhibition of the activity of G1-acting CDKs and thereby induces a G1 block in cell cycle progression. We also propose that I3C is mediating these effects through a putative cellular receptor ("receptor"). In contrast, the ER-dependent pathway is

mediated by the I3C acid-catalyzed product ICZ (indolo-[3,2*b*]carbazole) which binds to and activates the aromatic hydrocarbon receptor (AhR). The AhR transcriptionally activates cytochrome P4501A1 (CYP1A1)-dependent monooxygenase which inactivates estrone and thereby prevents the estrogen stimulated growth of breast cancer cells.

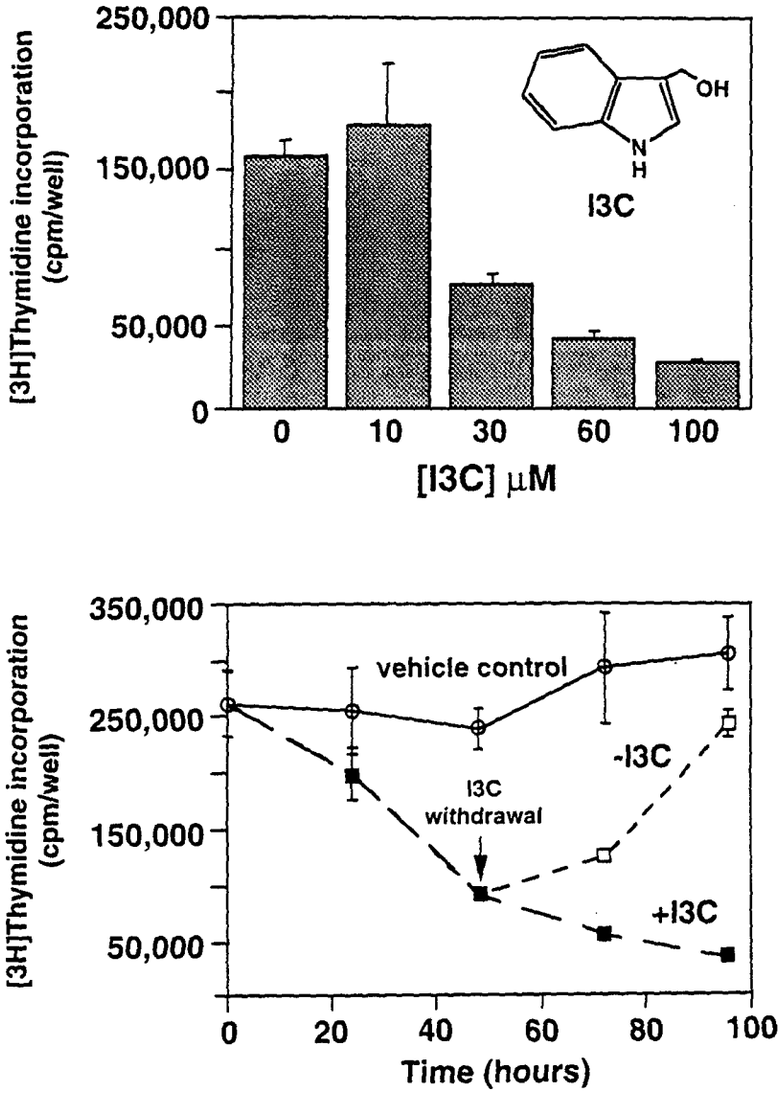

250,000
150,000
50,000
0
[3H]Thymidine incorporation (cpm/well)
OH
N
H
I3C
0
10
30
60
100
[I3C] μM
350,000
250,000
150,000
50,000
vehicle control
-I3C
I3C withdrawal
+I3C
0
20
40
60
80
100
Time (hours)

FIGURE 1

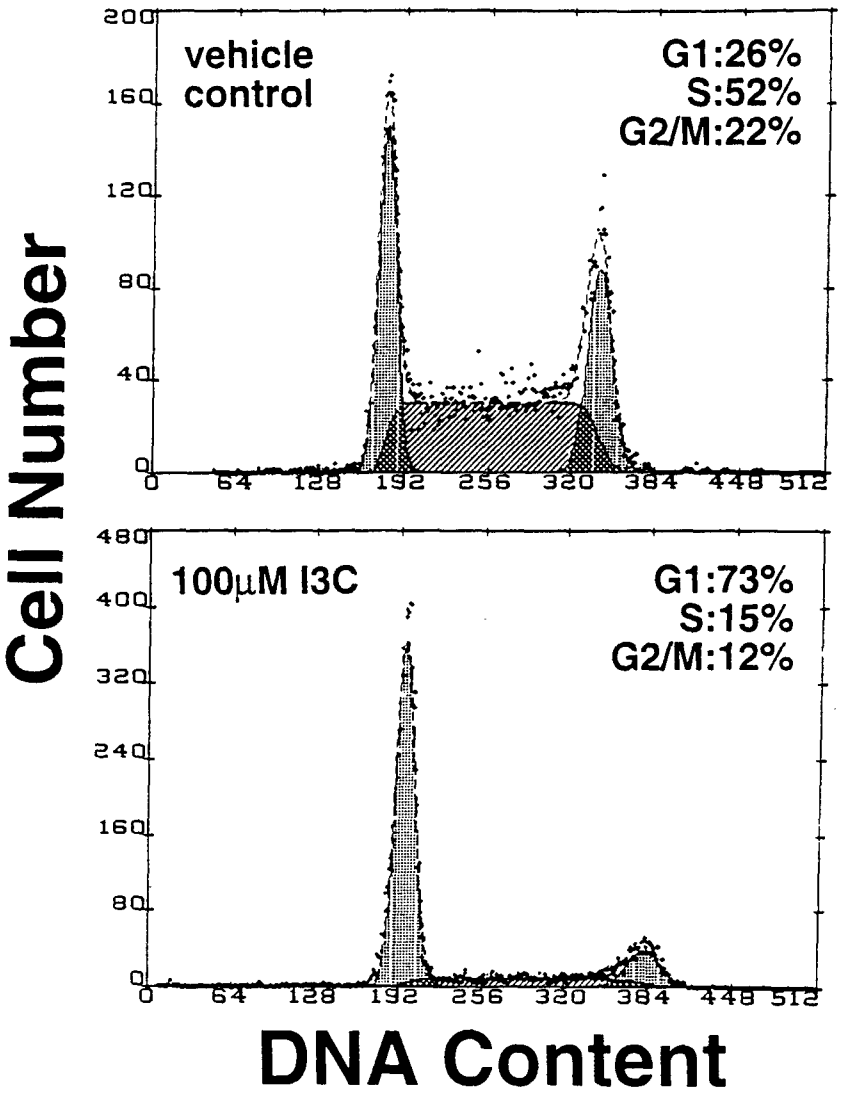
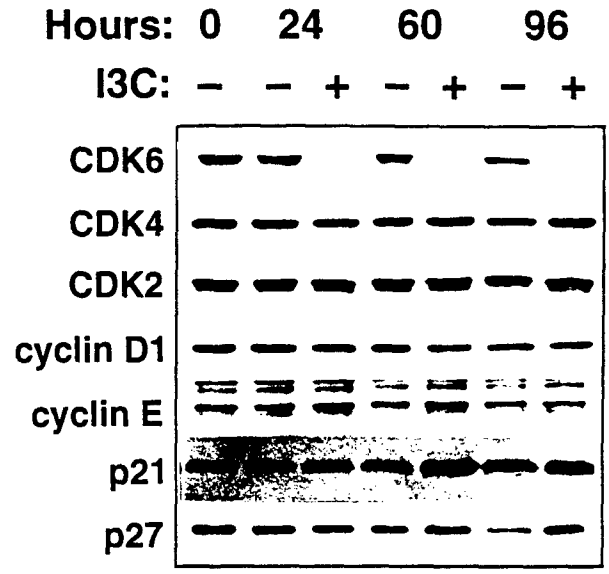

vehicle
control
G1:26%
S:52%
G2/M:22%
100μM I3C
G1:73%
S:15%
G2/M:12%
Cell Number
DNA Content
0
64
128
192
256
320
384
448
512
40
80
120
160
200
240
400
480

FIGURE 2


Hours: 0 24 60 96
I3C: – – + – + – +
CDK6
CDK4
CDK2
cyclin D1
cyclin E
p21
p27

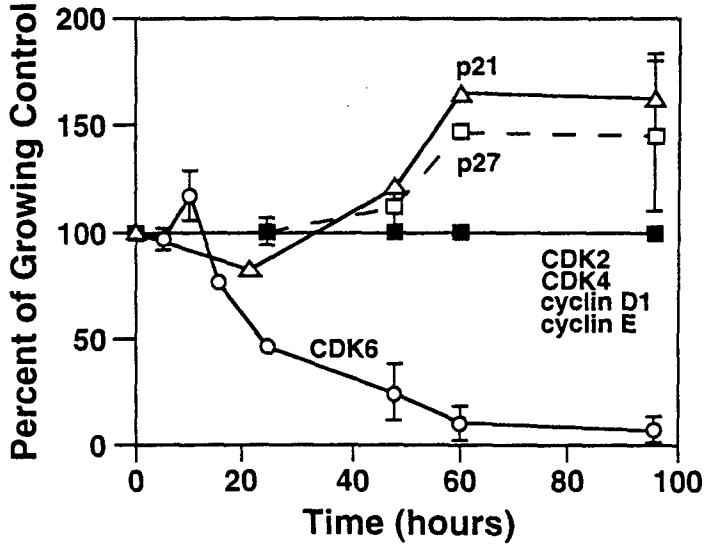

Percent of Growing Control
200
150
100
50
0
0
20
40
60
80
100
Time (hours)
p21
p27
CDK2
CDK4
cyclin D1
cyclin E
CDK6

FIGURE 3

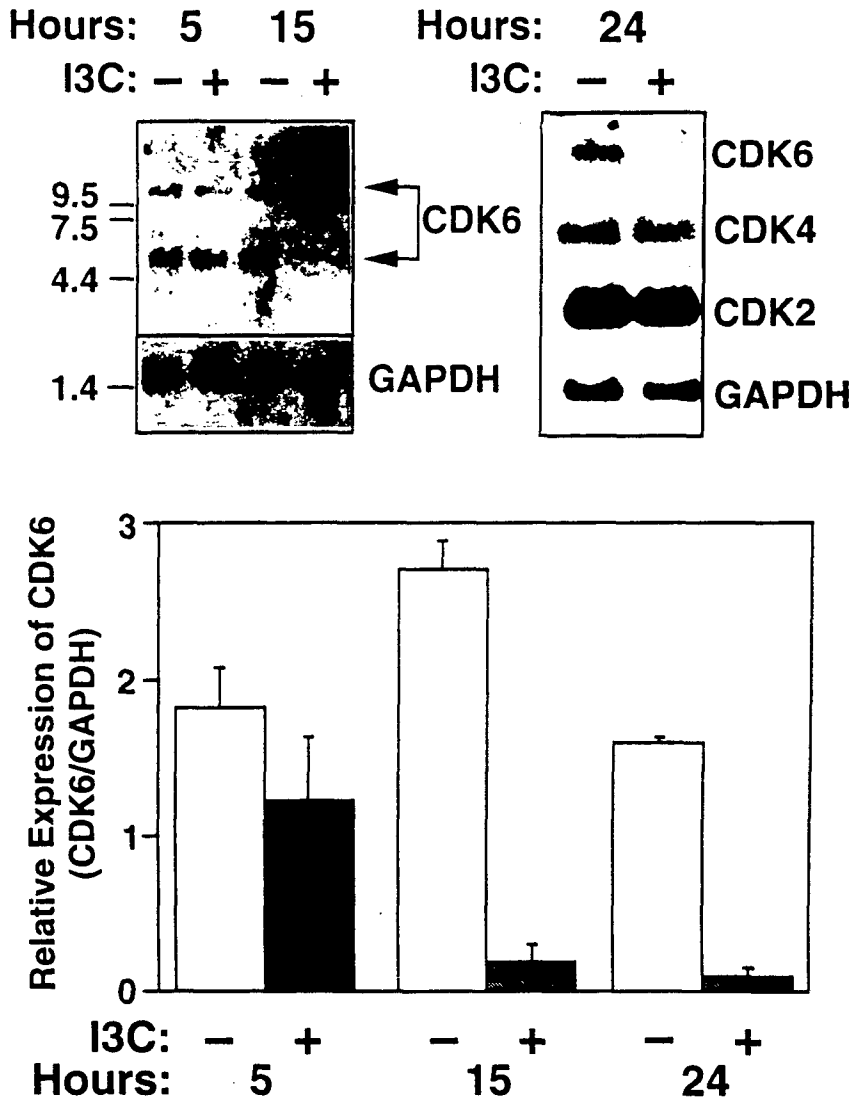

Hours: 5 15
I3C: – + – +
Hours: 24
I3C: – +
9.5
7.5
4.4
1.4
CDK6
GAPDH
CDK6
CDK4
CDK2
GAPDH
Relative Expression of CDK6
(CDK6/GAPDH)
3
2
1
0
I3C: – + – + – +
Hours: 5 15 24

FIGURE 4

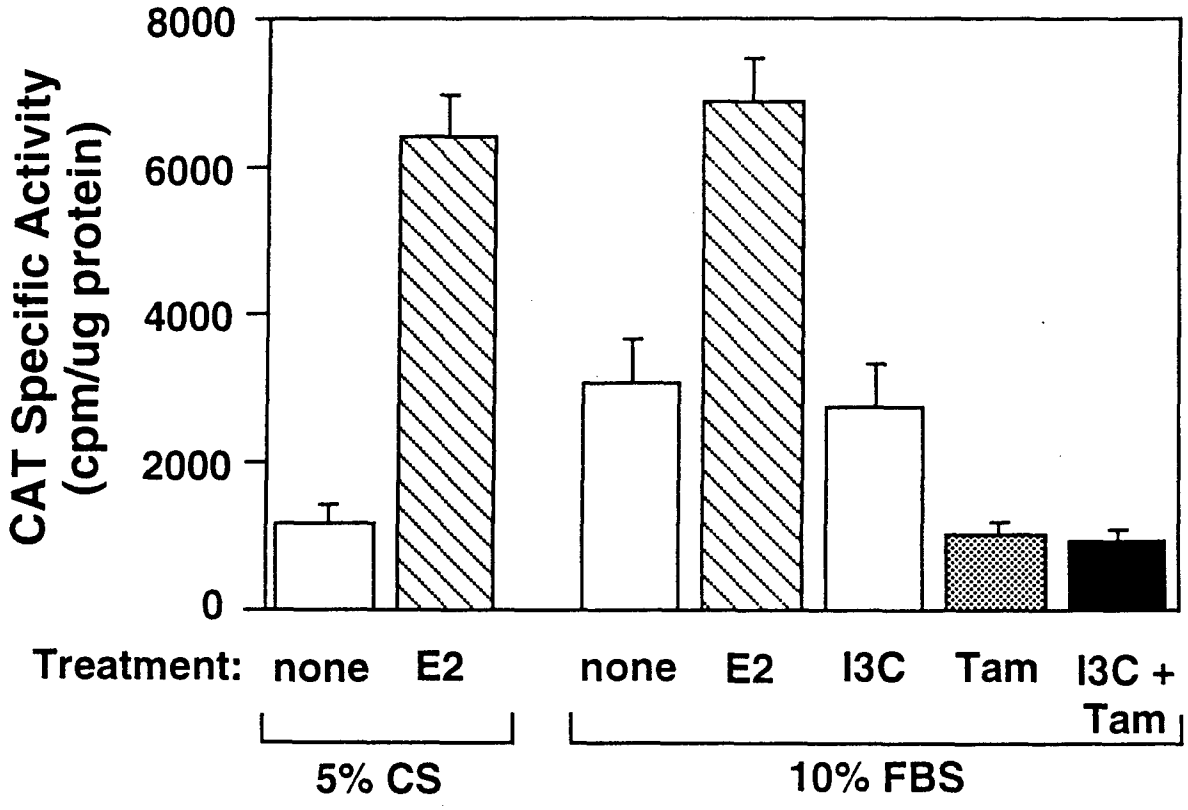

8000
6000
4000
2000
0
CAT Specific Activity
(cpm/ug protein)
Treatment:
none
E2
none
E2
I3C
Tam
I3C +
Tam
5% CS
10% FBS

FIGURE 5

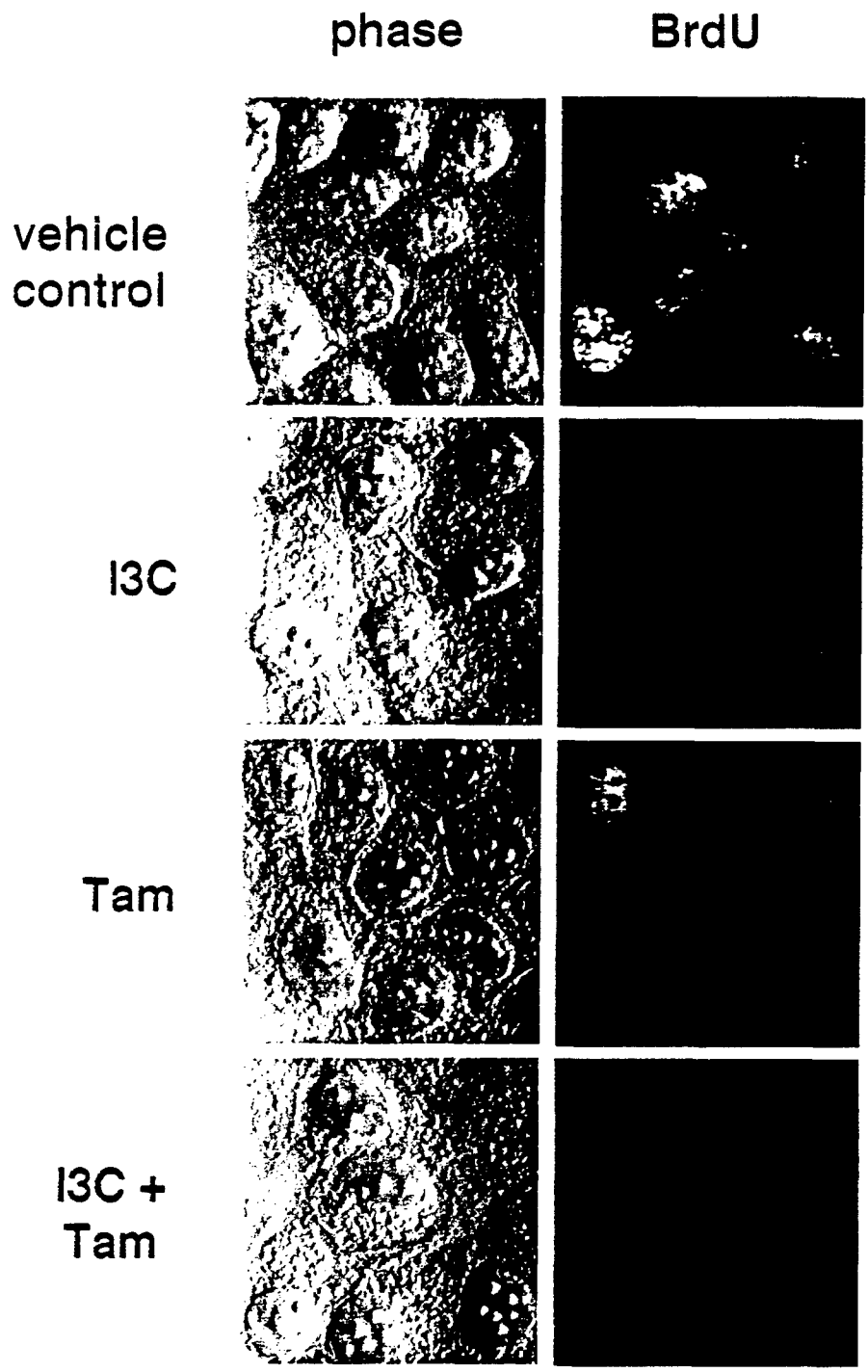


FIGURE 6

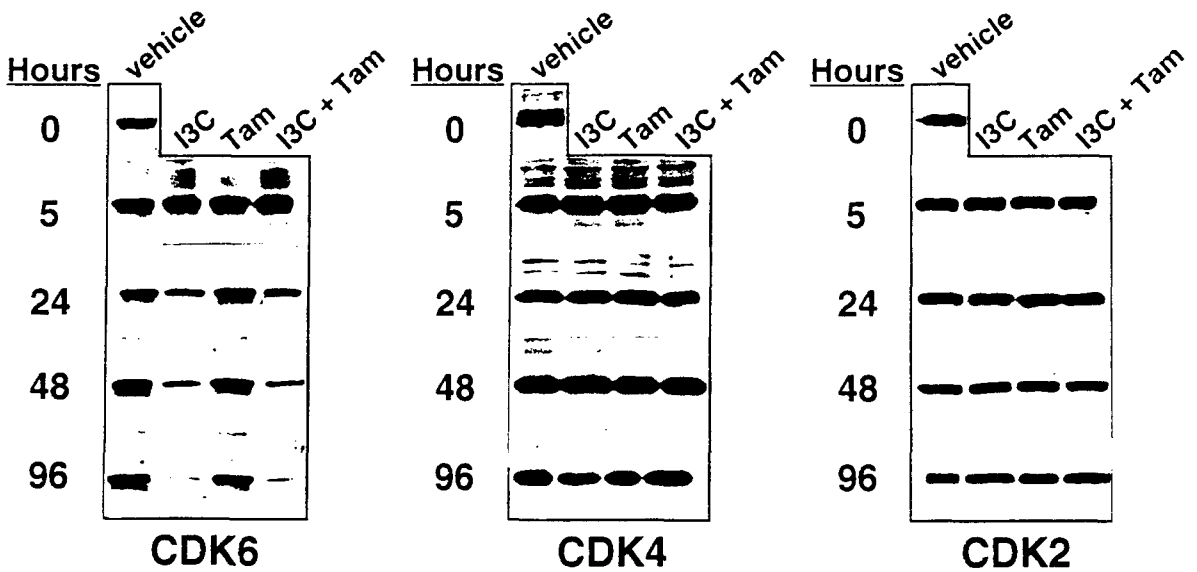


FIGURE 7

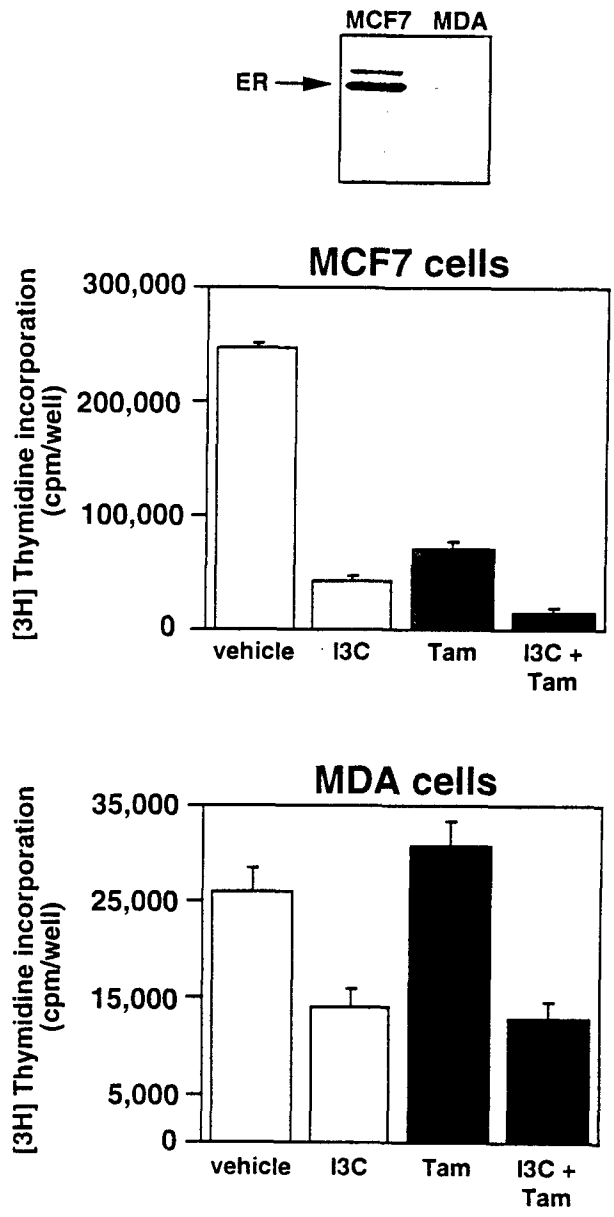

MCF7 MDA
ER
MCF7 cells
[3H] Thymidine incorporation (cpm/well)
300,000
200,000
100,000
0
vehicle
I3C
Tam
I3C + Tam
MDA cells
[3H] Thymidine incorporation (cpm/well)
35,000
25,000
15,000
5,000
0
vehicle
I3C
Tam
I3C + Tam

FIGURE 8

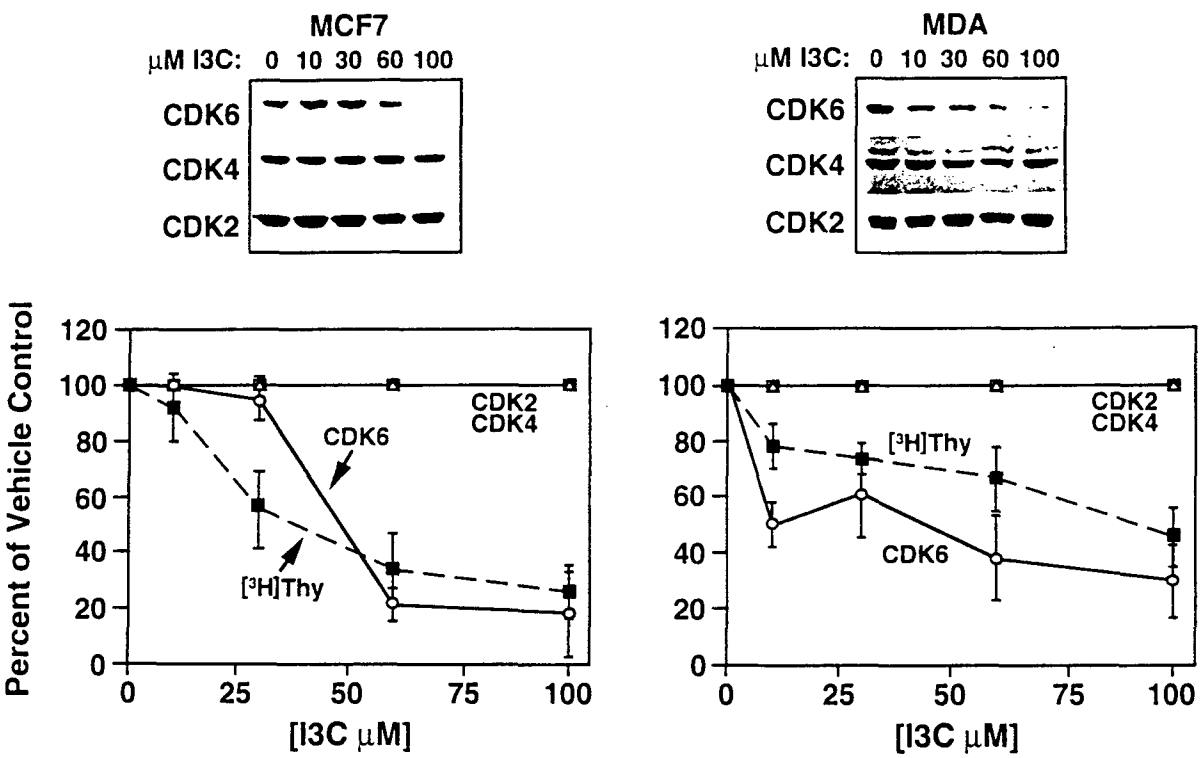

MCF7
μM I3C: 0 10 30 60 100
CDK6
CDK4
CDK2
MDA
μM I3C: 0 10 30 60 100
CDK6
CDK4
CDK2
Percent of Vehicle Control
120
100
80
60
40
20
0
0
25
50
75
100
[I3C μM]
CDK2
CDK4
CDK6
[3H]Thy
[3H]Thy
CDK2
CDK4
CDK6

FIGURE 9

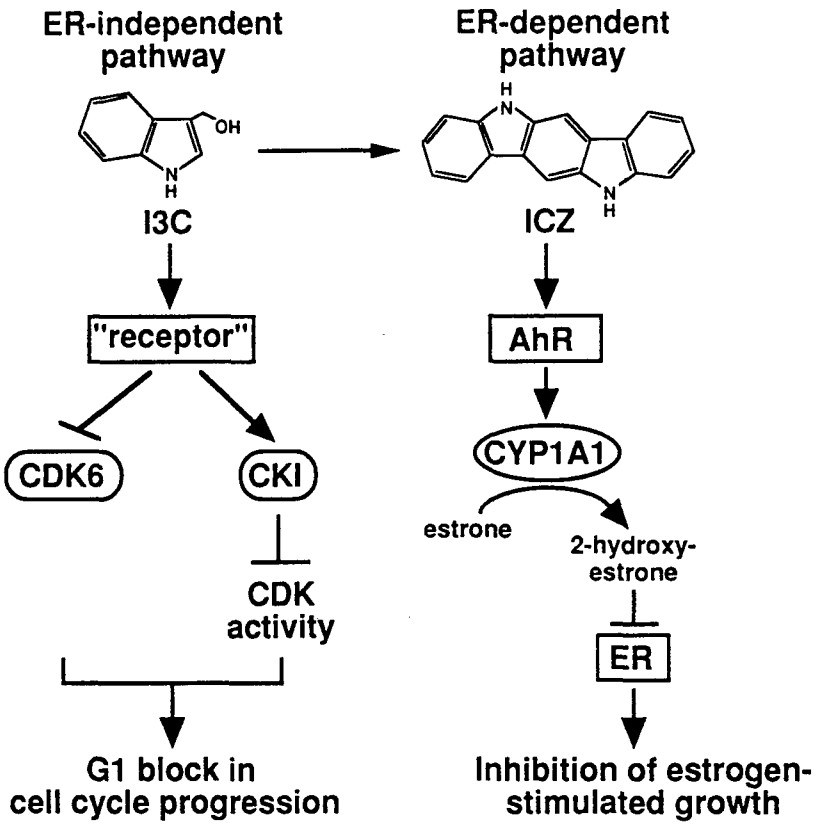

ER-independent pathway
ER-dependent pathway
OH
N
H
I3C
ICZ
"receptor"
AhR
CDK6
CKI
CYP1A1
estrone
2-hydroxy-estrone
CDK activity
ER
G1 block in cell cycle progression
Inhibition of estrogen-stimulated growth

FIGURE 10

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be Downgraded to Unlimited Distribution

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